AMP-Activated Protein Kinase

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AMP-ACTIVATED PROTEIN KINASE – A UBIQUITOUS SIGNALLING PATHWAY WITH KEY ROLES IN THE CARDIOVASCULAR SYSTEM

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ABSTRACT

The AMP-activated protein kinase (AMPK) is a key regulator of cellular and whole body energy homeostasis, which acts to restore energy homoeostasis whenever cellular energy charge is depleted. Over the last two decades, it has become apparent that AMPK regulates a number of other cellular functions and has specific roles in cardiovascular tissues, acting to regulate cardiac metabolism and contractile function as well as promoting anti-contractile, anti-inflammatory and anti-atherogenic actions in blood vessels. In this review, we will discuss the role of AMPK in the cardiovascular system, including the molecular basis of mutations in AMPK that alter cardiac physiology and the proposed mechanisms by which AMPK regulates vascular function under physiological and pathophysiological conditions.

The AMP-activated protein kinase (AMPK) is the central component of a signalling pathway that regulates the switch between anabolism and catabolism, as well as many other aspects of cell function[1-3]. In this review we focus on the physiological roles of AMPK within the cardiovascular system, although we will start by discussing general features that apply in all mammalian cell types. Readers are also referred to two other recent reviews of the role of AMPK in cardiovascular disease [4, 5].

STRUCTURE AND REGULATION OF AMPK

Occurrence of subunit isoforms

AMPK exists universally as heterotrimeric complexes containing catalytic α subunits and regulatory β and γ subunits, which occur as multiple isoforms (α1/α2; β1/β2; γ1/γ2/γ3) encoded by distinct genes. These could give rise in principle to twelve heterotrimeric combinations, although specific combinations appear to be favoured in specific cell types. For example, although skeletal muscle expresses mRNAs encoding all seven subunit isoforms, assays of immunoprecipitated isoforms suggest that AMPK activity in human skeletal muscle is accounted for by just three combinations: α1β2γ1, α2β2γ1 and α2β2γ3[6].

The domain organization of the seven AMPK subunit isoforms are shown in Fig. 1, and a representation of a crystal structure for the human α1β2γ1 heterotrimer[7] is shown in Fig. 2. Similar structures of the α2β1γ1[8] and α1β1γ1[9] complexes are available.

The α subunits

The α subunits contain a kinase domain (α-KD) at their N-termini, with the small N-lobe (yellow in Figs.1 and 2) and larger C-lobe (green) typical of all protein kinases. The substrate $Mg.ATP^{2-}$ binds in a deep cleft between these lobes, occupied in the structure in Fig. 2 by the non-specific kinase inhibitor staurosporine. Isolated α-KDs are only significantly active after phosphorylation by upstream kinases at a conserved threonine, usually referred to as Thr172. Thr172 phosphorylation is a good marker for AMPK activity, and Western blotting using phosphospecific antibodies against this site is often used as a semi-quantitative measure of AMPK activity. However, this ignores the effects of allosteric activation of AMPK, a problem that can be overcome by also addressing the phosphorylation state of a downstream target such as acetyl-CoA carboxylase (ACC).

The α-KD is followed by a small globular domain termed the autoinhibitory domain (AID, orange) that, when AMP is not bound to the γ subunit, binds to both the N- and C-lobes of the KD and thus causes inhibition[7]. The AID is connected to the C-terminal domain (α-CTD, red) by the α-linker (blue), a region of extended polypeptide that interacts with the γ subunit when AMP is bound at site 3 (as is the case in Fig. 2), thus pulling the AID away from this inhibitory position.

The β subunits

Two well-conserved regions within the β subunits are the C-terminal domain (β-CTD, silver-grey), which forms the "core" of the heterotrimeric complex, and the central carbohydrate-binding module (β-CBM, silver-blue). The β-CBM is related to carbohydrate-binding domains usually occurring in enzymes that metabolize starch or glycogen, which localize those enzymes on their polysaccharide substrate. Consistent with this, the β-CBM causes a proportion of AMPK in cells to bind to the surface of glycogen particles[10-12]. In all three structures of mammalian heterotrimers, the β-CBM interacts with the N-lobe of the α subunit KD, with its glycogen-binding site (defined by binding of the oligosaccharide β-cyclodextrin) at the top in the view of Fig. 2.

What is the function of glycogen binding by AMPK? The muscle and liver isoforms of glycogen synthase, which are also bound to glycogen particles, are physiological targets for AMPK[13, 14], and one function may be to co-localize AMPK with them. The β-CBM is also of interest because the cleft between it and the N-lobe of the α subunit forms the binding site for activators such as A769662 and 991[8]. This site has been termed the Allosteric Drug and Metabolite (ADaM) site[15], and is discussed further below.

The γ subunits

All γ subunits contain four tandem repeats of a sequence motif known as a CBS motif. In other proteins, pairs of CBS motifs often form binding sites for regulatory ligands containing adenosine[16]; in the AMPK-γ subunits they bind the regulatory nucleotides AMP, ADP or and ATP[17]. Two views of a structure for the γ1 subunit, containing three molecules of bound AMP[18], are shown in Fig. 3. The two pairs of repeats (CBS1:CBS2 and CBS3:CBS4) assemble in a pseudosymmetrical "head-to-head" manner. The γ subunit thus forms a structure like a flattened disc (seen from different faces in Fig. 3) with one CBS repeat in each quadrant, generating four potential ligand-binding clefts in the centre. However, one of these appears to be unused, perhaps because conserved

aspartate residues in CBS1, CBS3 and CBS4 that bind the ribose ring of adenine nucleotides in sites 1, 3 and 4 are absent from CBS2[18]. Interestingly, mutation of any of these three aspartate residues interferes with multiple effects of AMP on kinase activity[19], suggesting that occupancy of all three sites may be required for full activation.

Identity of upstream kinases

Identifying the upstream kinases that phosphorylate Thr172 was a difficult challenge, eventually solved by genome-wide biochemical screens that identified three upstream kinases in budding yeast [20, 21]. The mammalian kinases with catalytic domains most closely related to these were LKB1 and the Ca^{2+}/calmodulin-dependent kinase CaMKK2 (CaMKKβ), and evidence was soon obtained that both could act as physiological upstream kinases in mammalian cells[22-27]. The discovery that LKB1 was an upstream kinase for AMPK was particularly interesting, because LKB1 had previously been identified to be a tumor suppressor[28]. Phosphorylation of Thr172 by CaMKK2[25-27] represents a mechanism by which hormones that increase cytosolic Ca^{2+} can activate AMPK in the absence of energy stress.

GENERAL FEATURES OF AMPK REGULATION

Regulation by the canonical energy stress mechanism

It might be expected that a system that monitors cellular energy status would sense ATP and ADP but, interestingly, all metabolic enzymes known to directly monitor cellular energy charge (glycogen phosphorylase, 6-phosphofructo-1-kinase, fructose-1,6-bisphosphatase) primarily sense AMP and ATP, as does AMPK. The principal source of AMP in cells is thought to be the adenylate kinase reaction (2ADP ↔ ATP + AMP), which appears to operate close to equilibrium in most cells so that the AMP:ATP ratio will vary as the square of the ADP:ATP ratio[29]. The former is therefore a more sensitive indicator of falling energy status than the latter.

Binding of AMP activates AMPK by three complementary mechanisms, of which the first two are mimicked by ADP at higher concentration, while all three are antagonized by ATP: (i) inhibition of Thr172 dephosphorylation by protein phosphatases; (ii) promotion of Thr172 phosphorylation by LKB1; and (iii) allosteric activation[30, 31]. The structural model shown in Fig. 2 suggests a mechanism, for which there is now supporting evidence[7], to explain mechanisms (i) and

(iii). When AMP is bound at site 3, the α-linker interacts with the surface of the γ subunit containing that site (Fig. 2)[7, 32]. AMPK heterotrimers contain two rather distinct regions, the "catalytic module" (β-CBM, KD, AID, top/front section in Fig. 2) and the "nucleotide-binding module" (γ subunit, β-CTD, α-CTD, bottom/rear section in Fig. 2). The "hinge" connecting them is the α-linker, and release of the α-linker on binding of ATP rather than AMP at site 3 is envisaged to allow the two modules to move apart, causing the AID to rotate back into its inhibitory position behind the α-KD. This conformational change would also increase accessibility of Thr172 to protein phosphatases, which in the AMP-bound conformation of Fig. 2 is around the back, located in a deep cleft between the two modules. This model therefore explains not only how AMP binding at site 3 causes allosteric activation, but also why it protects against Thr172 dephosphorylation, with binding of ATP antagonizing both effects.

Multiple mechanisms of pharmacological activation of AMPK

A selection of compounds commonly used to activate AMPK experimentally are listed in Table 1. Those in Class 1, including the antidiabetic drug metformin and berberine (derived from traditional Chinese medicine) inhibit Complex I of the mitochondrial respiratory chain, while those in class 2, including 2-deoxyglucose, inhibit glycolysis. Both classes activate AMPK indirectly by increasing cellular AMP:ATP ratios[33]. These compounds are frequently used to activate AMPK experimentally, and studies utilizing them are described later in this review. However, because they work by depleting cellular ATP, they should not be regarded as specific AMPK activators and any results obtained with them should ideally be followed up using genetic approaches, such as the use of AMPK knockouts.

The third class of activator includes the widely used compound 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), a nucleoside that is taken up into cells and converted to the equivalent nucleotide, ZMP. An important caveat here is that although ZMP is an AMP analog that mimics the effects of AMP to activate AMPK, it is about 50-fold less potent than AMP itself[34]. Because ZMP accumulates to millimolar concentrations inside cells AICAR does cause AMPK activation, but ZMP also has off-target effects. For example, it is known to mimic the effects of AMP on phosphorylase[35] and fructose-1,6-bisphosphatase[36] as well as AMPK. Another problem with AICAR is that it is an adenosine analogue and, while it does not appear to bind directly to

adenosine receptors, in incubated cell systems it competes with endogenous adenosine for reuptake into cells by adenosine transporters, so can have adenosine-like effects [37].

A more specific activator that works via a related mechanism (class 4) is C13, which is taken up by cells and converted by cellular esterases to the AMP analog C2, a very potent AMPK activator[38] although only for α1- and not α2-containing complexes[39]. Despite its selectivity for the α1 isoform, C2 binds to the γ subunit. Although the AMP and C2 binding sites overlap, they are not identical[40].

The other molecules of choice for selective activation of AMPK are those binding at the ADaM site located between the β-CBM and the N-lobe on the α subunit, such as A769662 and 991 (class 5)[8]. All compounds binding this site are more potent activators of complexes containing AMPK-β1 rather than β2, and most are essentially β1-selective (Table 1). Using AMPK phosphorylated on Thr172, these activators cause a modest degree of allosteric activation (up to 4-fold) and, by inhibiting dephosphorylation, also promote net Thr172 phosphorylation[41, 42]. More remarkably, they cause a much larger allosteric activation (up to 65-fold) of AMPK that is not phosphorylated on Thr172, and this effect is synergistic with AMP[43]. At present, almost all of the activators known to bind at this site are synthetic compounds derived from high-throughput screens, although there is much speculation in the field that there is a naturally occurring ligand, hence the appearance of "metabolite" in its name[15]. However, no natural ligands occurring in animal cells that bind the ADaM site have yet been found. Salicylate, the natural plant product from which acetyl salicylic acid (ASA, aspirin) was derived, does activate AMPK by binding this site[9, 44].

It should also be noted that there are currently no specific pharmacological inhibitors of AMPK; compound C (dorsomorphin) is sometimes claimed to be a specific inhibitor, but this is not the case[45].

TARGETS AND PATHWAYS DOWNSTREAM OF AMPK

Catabolic effects switched on by AMPK

Once activated by energy stress, AMPK switches on catabolic pathways generating ATP, while switching off anabolic pathways and other processes consuming ATP, thus acting to restore energy homeostasis. This topic has been discussed in more detail in previous reviews[1-3]. Examples of catabolic processes acutely switched on include cellular glucose uptake mediated by GLUT1 and GLUT4. Activation of GLUT1 may occur via phosphorylation of the thioredoxin interacting protein

TXNIP[46], while enhanced GLUT4 translocation to the plasma membrane in muscle appears to occur, at least in part, by phosphorylation of TBC1D1, which modulates trafficking of GLUT4-containing vesicles[47]. AMPK activation also enhances GLUT4 expression[48], in part via phosphorylation of the histone deacetylase HDAC5, promoting binding of 14-3-3 proteins and consequent retention of HDAC5, a transcriptional inhibitor, in the cytoplasm[49, 50]. AMPK can also cause a short-term activation of glycolysis via phosphorylation of PFKFB2[51] and PFKFB3[52], isoforms of the bifunctional enzyme that synthesizes and breaks down fructose-2,6-bisphosphate, a key allosteric activator of 6-phosphofructo-1-kinase and hence glycolysis. PFKFB2 is expressed in the heart, while PFKFB3 occurs as an "inducible" form whose expression in monocytes and macrophages is induced by inflammatory mediators such as lipopolysaccharide[53]. Phosphorylation of PFKFB2 or PFKFB3 at equivalent sites near their C-termini increases the synthesis of fructose-2,6-bisphosphate and hence promotes glycolysis during hypoxia in heart and in activated monocytes/macrophages, respectively. This may enhance survival of these cells during periods of hypoxia or ischemia.

Although AMPK can therefore activate glucose uptake and glycolysis in specific cell types, in the longer term it tends to promote instead the more glucose-sparing and energy-efficient oxidative metabolism. In skeletal muscle[54, 55] and liver[56], AMPK activates fatty acid oxidation by inhibiting the ACC1/ACC2 isoforms of acetyl-CoA carboxylase to reduce malonyl-CoA, an inhibitor of the uptake of fatty acids into mitochondria. AMPK promotes the expression of TCA cycle enzymes[57], as well as mitochondrial biogenesis, which it achieves by increasing the expression/activity of the transcriptional co-activator PGC-1α, either by direct phosphorylation triggering a positive feedback effect on its own expression[58], or by enhancing its deacetylation by SIRT1[59]. PGC-1α acts as a co-activator for several transcription factors involved in mitochondrial biogenesis and oxidative metabolism, including myocyte enhancer factor-2 (MEF2), nuclear respiratory factors-1/-2 (NRF1/2), and PPAR-α and –δ[60]. Despite this evidence that AMPK promotes oxidative metabolism, in mice with a skeletal/cardiac muscle-specific knockout of both AMPK-β subunits, although the mice displayed evidence of dilated cardiomyopathy the rate of glucose and fatty acid oxidation in hearts perfused under normoxic conditions *ex vivo* was normal[61].

A final catabolic pathway switched on by AMPK is autophagy, brought about via direct phosphorylation of the protein kinase that triggers that process, ULK1[62]. By triggering digestion of

cellular contents, autophagy may be critical in enhancing cell survival during periods of acute nutrient starvation, such as during ischemia in cardiac muscle. However, it has also been suggested that excessive autophagy might also contribute to cell damage during ischemia/reperfusion[63].

Anabolic pathways switched off by AMPK

As well as its classical roles in inhibiting fatty acid and sterol synthesis[34], AMPK inactivates key enzymes and regulatory proteins involved in triglyceride/phospholipid synthesis[64], glycogen synthesis[13, 14], rRNA synthesis[65], and protein synthesis. The latter is inhibited both at the elongation step by phosphorylation and activation of elongation factor-2 (EF2) kinase[66], and at the initiation step by inactivation of the mechanistic target-of-rapamycin complex-1 (mTORC1) by multiple mechanisms[67, 68]. By inhibiting mTORC1, AMPK would also be expected to oppose hypertrophy in organs such as the heart, which in most cases is considered to be a deleterious process.

ROLE OF AMPK IN THE HEART

Role in the response to cardiac ischemia

It was reported in 1995 that AMPK was activated by no-flow ischemia in perfused rat hearts, an effect associated with high rates of fatty acid oxidation during reperfusion[69]. AMPK is also activated in the heart by increased workload[70]. The role of AMPK in cardiac ischemia has subsequently been addressed using mouse models where AMPK is down-regulated or absent. The first was a transgenic model where an inactive AMPK-α2 subunit was expressed in both skeletal and cardiac muscle. By competing with endogenous α subunits for available β and γ subunits, the over-expressed inactive α2 subunit down-regulates endogenous α1 and α2, and thus acts as a dominant negative mutant. There was no major phenotype under unstressed conditions, but during low-flow ischemia there was a failure to enhance glucose uptake and glycolysis, while during subsequent reperfusion there was less fatty acid oxidation, lower ATP levels and poorer recovery of contractile function[71]. The degree of damage to the myocardium was also greater, suggesting that AMPK exerts a cardioprotective effect overall, even though stimulation of fatty acid oxidation during reperfusion may be deleterious[69]. Using the same model, evidence was obtained that AMPK was required for increased autophagy during ischemia[72].

Next to be studied were conditional knockouts of the upstream kinase LKB1 in both skeletal and cardiac muscle. The basal activity of AMPK-α2 complexes in the heart was completely abolished, and did not increase in response to no-flow ischemia or anoxia, correlating with a complete lack of ACC phosphorylation at the AMPK site. The AMP:ATP and ADP:ATP ratios were also elevated to a greater extent during no-flow ischemia than in control hearts, confirming that AMPK was protecting the cells against energetic stress. Surprisingly, the activity of AMPK-α1 complexes was almost unaffected by LKB1 knockout, and still increased in response to ischemia or anoxia[73]. Broadly similar results were obtained in studies of perfused hearts from mice with a whole body knockout of AMPK-α2[74]. Interestingly, left ventricular hypertrophy induced by phenylephrine was greatly accentuated in the hearts from AMPK-α2 knockout mice; this may have been due to less restraint on the mTORC1 pathway, because the basal phosphorylation of p70S6K at the mTORC1 site was elevated, although it did not increase further upon phenylephrine treatment as in the controls[75].

Mutations in γ2 and γ3 subunits causing heart disease and altered glycogen content

The only mutations in any of the seven genes encoding AMPK subunits clearly shown to cause human pathology are those in *PRKAG2*, encoding AMPK-γ2. These are associated with multiple cardiac disorders, specifically: (i) ventricular pre-excitation; (ii) excessive glycogen storage in cardiomyocytes; (iii) cardiac hypertrophy. Although rare, the *PRKAG2* mutations are autosomal dominant in effect and therefore occur frequently in affected families. They are invariably missense mutations causing amino acid replacements, of which at least 14 have now been reported (Table 2). The affected residues are perfectly conserved between human γ1, γ2 and γ3, and occur in all four CBS repeats. The location of the residues affected in γ2, and of the three bound molecules of AMP, are mapped in Fig. 3 onto a structure for γ1[18]. Interestingly, six of the mutations (R302Q, H383R, R384T, H530R, R531G and R531Q) affect basic residues whose side chains directly interact with the phosphate groups of one or more molecules of AMP or ATP, while two others also interact either directly (S548) or indirectly (T400) with AMP[18]. Other affected residues lie in more peripheral regions of the γ subunit, and it is less obvious why their replacement should affect function. In the context of bacterially expressed CBS repeats from AMPK-γ2, the R302Q, H383R,

T400N, and R531G mutations all reduced the affinity for ATP as well as AMP, with the severity of the effects increasing in the order R302Q<H383R<T400N<R531G[16]. When expressed in mammalian cells as α1β1γ2 heterotrimers, these mutations also reduced allosteric activation by AMP, with the R531G mutation abolishing AMPK activation completely[16, 76]. The R384T and R531Q mutations were shown later to cause severe effects on AMPK function, similar to or even greater than R531G[77, 78]. In a comparison of the R531G and R531Q mutations expressed as α1β1γ2 heterotrimers in HEK-293 cells, these mutations not only abolished allosteric activation by AMP, but also caused significant increases in basal Thr172 phosphorylation and activity[78]. This was particularly clear in cells stably expressing the R531G mutant, when clones could be selected in which the level of expression of the WT and mutant was identical; the R531G mutant consistently had a 2-fold higher basal Thr172 phosphorylation and activity although, unlike the wild type, it was completely insensitive to further activation by agents that increase cellular AMP/ADP[33].

Some of the mutations, such as R302Q, can cause relatively mild symptoms so that the patients may not present in the clinic until early adulthood[79]. By contrast, patients with the R531G mutation develop quite severe symptoms during childhood[80], while the R384T[77] and R531Q[78] mutations were only detected *post mortem* in neonates who had died within weeks of birth, and appeared to be *de novo* mutations because neither parent was affected. Consistent with the idea that R531G, R531Q and R384T cause particularly severe forms of the disease, these were the mutations with the largest effects on binding of AMP and ATP to the γ subunit[16, 77, 78]. It is also interesting that R531 and R384 are involved in the binding of AMP and/or ATP at the critical site, site 3.

What causes the cardiac sequelae of the *PRKAG2* mutations? The reduction of AMP binding to the γ subunit causes reduced activation by AMP and is a loss-of-function effect, whereas the reduction of ATP binding may be responsible for the increased basal activity of the R531G and R531Q mutants and is a gain-of-function effect. Since these mutations are dominant, it seems likely that it is the gain-of-function effect, increased basal activity, that causes most of the pathology; the loss-of-function effect may be compensated for by γ1, which is the major isoform expressed in heart, at least in rodents[81]. This is an important conclusion because pharmaceutical companies are developing AMPK activators to combat Type 2 diabetes, and it suggests that activation of γ2-containing complexes in the heart might have deleterious effects, as seen with these mutations.

Why does increased basal activity of γ2-containing complexes cause these cardiac phenotypes? AMPK is known to promote glucose uptake in cardiac muscle[71], so increased basal activity would be expected to cause a high basal uptake, even in the absence of a real demand for glucose. There is good evidence for this scenario from studies of transgenic mice over-expressing the N488I mutation in the heart, which develop ventricular pre-excitation and cardiac hypertrophy similar to the human disorder[82]. By 50 days of age, they have a 20-fold increase in cardiac glycogen compared with controls, accompanied by higher rates of glucose uptake and glycogen synthesis but lower rates of lactate production, suggesting that increased glucose uptake is being directed into glycogen synthesis rather than glycolysis. Glycogen synthase was also much more highly phosphorylated in the transgenic mice (presumably due to the high basal AMPK activity), but cellular glucose-6-phosphate (G6P, an allosteric activator of glycogen synthase that over-rides the effects of phosphorylation) was also elevated >3-fold. Satisfying confirmation of this interpretation came when the N488I mice were crossed with knock-in mice carrying a mutation that renders glycogen synthase insensitive to G6P. The presence of the G6P-insensitive glycogen synthase reversed the high glycogen phenotype of the N488I mice and rescued the ventricular pre-excitation but not the cardiac hypertrophy, suggesting that the former but not the latter is secondary to increased glycogen content[83]. Supporting the idea that hyper-activation of the mTORC1 pathway was the cause of hypertrophy, mTORC1 targets such as p70S6K and 4EBP1 were more highly phosphorylated in the N488I mice, while treatment with rapamycin reduced, without completely preventing, the hypertrophy. Increased mTORC1 in the N488I mice is actually rather counter-intuitive, because AMPK is normally thought to inhibit that pathway[67, 68]. However, a recent study has suggested that AMPK can activate mTORC1 under certain circumstances by sustaining the supply of amino acids via autophagy[84].

Why does abnormally high glycogen content lead to ventricular pre-excitation? During foetal development, the atria and the ventricles become separated by the growth of a fibrous layer called the *annulus fibrosis*, which ensures that the only electrical connection between the two chambers is via the atrioventricular node. In N488I mice the annulus fibrosis appeared to be thin and disrupted in places, and it was suggested that the presence of glycogen-containing vacuoles in myocytes disrupts its formation during fetal development, causing abnormal electrical connections between the atria and ventricles[85].

Most mouse studies of γ2 mutations have involved transgenic mice over-expressing the mutations in human γ2. Although these mice do display the key clinical features of the human syndrome, and mice expressing wild type γ2 from the same promoters were used as controls, they are not perfect models because γ2 is being over-expressed. Recently, three knock-in mouse models, in which mutations in the mouse gene equivalent to human R302Q, N488I or R531G are expressed globally, were studied[86, 87]. There was evidence for increased basal AMPK activity in liver and/or muscle of all three strains. The N488I and R531G mice displayed ventricular pre-excitation and modest increases in heart weight, which was associated with large increases in glycogen content in hearts of R531G but not the N488I mice. Both N488I and R531G mice displayed resistance to obesity and hepatic steatosis induced by high-fat diet, but, unlike the N488I mice, the R531G mice also displayed impaired renal function associated with glycogen accumulation, cyst formation, and inflammation and apoptosis in the kidney, particularly when on a high-fat diet[87], By contrast, in the R302Q mice there was no obvious cardiac phenotype, but the homozygotes developed marked obesity as they aged, with smaller effects in heterozygotes. Obesity appeared to be due mainly to increased food intake through enhanced action of ghrelin, whose effects are mediated by AMPK activation via the CaMKK2 pathway in the hypothalamus[88, 89]. The homozygotes were also hypoinsulemic, apparently due to reduced glucose-stimulated insulin secretion from the pancreas. Overall, the phenotypes of these mice with knock-in mutations in the *PRKAG2* gene showed surprising variability. Interestingly, heterozygous human carriers of the R302Q mutation, who have a relatively mild cardiac phenotype, also display some evidence of increased obesity, as well as higher fasting glucose and glycated haemoglobin (HbA1c) and lower insulin levels than unaffected siblings[86]. These non-cardiac features of the R302Q mutation only became evident following studies of the mouse model.

Finally, all of the residues mutated in AMPK-γ2 are also conserved in γ1 and γ3 (Table 2). It is interesting that none of them have yet been reported to be mutated in the human γ1 gene. However, mutations equivalent to R302Q have been found in both pigs and humans in the γ3 isoform, which is predominantly expressed in skeletal muscle[81]. In human muscle, γ3 appears to be present exclusively as the α2β2γ3 complex, which is the only form of AMPK that is activated during exercise[6]. An R200Q mutation (equivalent to R302Q in human γ2) was found to be a relatively common dominantly acting genetic variant in Hampshire pigs, and was associated with a high

glycogen content in skeletal muscle; although adversely affecting meat quality, it did not cause obvious clinical problems[90]. Interestingly, in a screen of around 1500 humans, an R225W mutation (R225 being the human equivalent of R302) was found in two apparently unrelated individuals, once again not associated with any obvious clinical defects; muscles from humans with this mutation had a higher basal AMPK activity, 2-fold higher glycogen content and lower triglyceride content[91]. In an *in vitro* study of myotubes from R225W carriers, they had 3-fold higher mitochondrial content and oxidative capacity, and 2-fold higher basal glucose uptake and glycogen synthesis rates than matched controls. The R225W subjects also had a remarkable resistance to fatigue during isometric contractions of the quadriceps[92], suggesting that this mutation might confer an advantage during endurance exercise because of the high glycogen content.

ROLE OF AMPK IN THE VASCULATURE

In addition to the heart, it has become clear that AMPK has an important role in regulating vascular function, with distinct roles in the functions of vascular endothelial cells (ECs), smooth muscle cells (VSMCs), adventitial cells and vascular immune cells.

Regulation of AMPK in endothelial and vascular smooth muscle cells

AMPK-α1 accounts for the majority of total AMPK activity in ECs[93-95], yet specific down-regulation of AMPK-α2 still has marked effects[96, 97]. A wide variety of physiological stimuli have been reported to activate endothelial AMPK, including hypoxia, low glucose and shear stress[98-100], adiponectin[101], angiotensin II and ghrelin[102, 103]. In addition, CaMKK2 mediates AMPK activation by vasoactive molecules including thrombin, VEGF, sphingosine-1-phosphate, bradykinin and estrogen[104-108], suggesting that any stimulus that increases Ca^{2+} in ECs will activate AMPK. Finally, a number of widely used hypoglycemic (metformin, thiazolidinediones, salicylate, DPP4 inhibitors and liraglutide)[109-112] and hypocholesterolemic (statins and fenofibrate)[113, 114] drugs activate AMPK in cultured ECs.

Physiological signals that *inhibit* AMPK in ECs include high nutrient concentrations[115-117], yet the mechanisms by which this inhibition of AMPK in ECs occurs are unclear, although increased PP2A-mediated dephosphorylation of AMPK has been proposed[115]. Endothelial AMPK may therefore be suppressed by the high levels of nutrients associated with obesity and insulin resistance. As protein kinase C (PKC) activation is associated with over-nutrition, it is interesting

that PKC-mediated inhibitory phosphorylation of Ser487 on AMPK-α1 has been recently reported in ECs[118]. Conversely, the very first study of AMPK function in ECs demonstrated that AICAR stimulated fatty acid oxidation[119] and AMPK activation was subsequently found to normalise impaired fatty acid oxidation and insulin signaling due to high glucose[120]. Stimulation of fat oxidation may partly underlie the effect of AMPK to antagonize palmitate-mediated endothelial dysfunction, thus protecting against lipotoxicity.

As with ECs, AMPK-α1 accounts for the majority of AMPK activity in murine and human VSMCs[121, 122], and low glucose, adiponectin, estradiol and metformin all activate AMPK[123-126]. Angiotensin II has also been reported to acutely activate AMPK[127], although prolonged activation had no effect[128]. Similar to ECs, high glucose inhibits AMPK in VSMCs, and IGF-1 also inhibits AMPK, most likely via inhibitory phosphorylation of AMPK-α1 by Akt[129]. Several studies have demonstrated that culture in phosphate/β-glycerophosphate, used to examine VSMC calcification as described later, also inhibits AMPK[130, 131].

AMPK and endothelial NO synthesis

Endothelium-derived NO is a key regulator of vascular function, stimulating VSMC relaxation whilst inhibiting pro-inflammatory signaling, leukocyte adhesion, platelet aggregation, VSMC proliferation and migration associated with pathological vascular remodelling and atherosclerosis[132]. The first indication of a specific vascular role for AMPK came when AMPK was shown to phosphorylate at Ser1177 and activate endothelial NO synthase (eNOS), both in cell-free assays and in ECs[93, 133]. Multiple mechanisms regulate eNOS activity, including Ca^{2+}/calmodulin binding, allosteric and protein:protein interactions, co-factor and substrate availability, phosphorylation, and subcellular localisation[132]. In addition to Ser1177, AMPK phosphorylates eNOS at Ser633, required for AICAR-stimulated NO synthesis in HEK293 cells[134]. NO synthesis requires dimerization of eNOS and sufficient tetrahydrobiopterin (BH_4), in whose absence eNOS generates superoxide instead[132]. Another mechanism by which AMPK improves NO synthesis may be by enhancing BH_4 levels, as AMPK prevents degradation of GTP cyclohydrolase I, which is the rate-limiting enzyme in BH_4 synthesis[135]. AMPK can therefore act by multiple mechanisms to stimulate NO production (Fig. 4), although endothelial AMPK activation is not always associated

with eNOS phosphorylation or NO synthesis[94, 136]. AMPK may also mediate some of the endothelial effects of NO, because NO donors activate AMPK in ECs[96].

Endothelial AMPK and reactive oxygen species

Inappropriate levels of reactive oxygen species (ROS), in particular superoxide anions generated in response to high concentrations of glucose, lipids and proinflammatory cytokines by NAD(P)H oxidase (Nox), uncoupled eNOS or mitochondrial respiratory chain complexes, have been implicated in vascular disease[97, 135, 137, 138]. Superoxide reacts with NO to form peroxynitrite, reducing NO bioavailability[132]. AMPK activation in ECs has been widely demonstrated to inhibit ROS formation, increase antioxidant defences and promote mitochondrial biogenesis. Down-regulation of AMPK in ECs increased activity and expression of Nox[97], whereas AMPK-dependent inhibition of Nox1/2 translocation to the plasma membrane has also been reported[109]. The mechanism by which AMPK inhibits Nox remains unclear, but may be secondary to reduced PKC-mediated Nox activation, or NFκB-mediated Nox transcription[97, 109]. AMPK-dependent inhibition of mitochondrial ROS formation has also been reported in ECs maintained in high glucose [137], whereas AMPK-mediated stimulation of BH_4 synthesis, as described above, prevents uncoupling of eNOS and superoxide formation[135]. Several groups have reported that AMPK-mediated inhibition of ROS in ECs is associated with increased levels of the antioxidant enzymes superoxide dismutase-2 (SOD2), catalase and thioredoxin [137, 139, 140]. In ECs, AMPK activation reduces ER stress[141], which is tightly linked to oxidative stress and inflammation, whereas silencing of AMPK increases markers of ER stress[95]. Taken together, AMPK activation acts via multiple mechanisms to suppress chronic ROS synthesis in ECs, limiting their damaging actions as well as the sequestration of NO (Fig 4).

AMPK and vascular cell inflammation

The development of endothelial dysfunction and cardiovascular diseases is associated with elevated TNFα, IL-1β and IL-6[142]. TNFα and IL-1β stimulate activation of NFκB and the Jun N-terminal kinase (JNK) pathway, while IL-6 signals via Janus kinases (JAKs) leading to phosphorylation of signal transducer and activation of transcription (STAT) proteins[143]. The anti-inflammatory actions of AMPK in ECs were first described when AMPK activation was shown to suppress palmitate- or TNFα-stimulated NFκB activity[117]. Under basal conditions, NFκB is in an inactive form in the

cytoplasm due to binding to IκB whereas, after cytokine stimulation, IκB phosphorylation by IκB kinase (IKK) targets it for degradation, allowing NFκB-mediated transcription of proinflammatory cytokines, adhesion molecules and chemokines[143, 144]. NO inhibits endothelial NFκB activity[145], suggesting that AMPK-stimulated NO synthesis would inhibit NFκB, although NO donors do not effectively suppress NFκB in ECs with reduced AMPK activity[96]. AMPK-α2 has been reported to phosphorylate IKKβ *in vitro*, inhibiting IκB phosphorylation and NFκB activation, with reduced IL-1β-stimulated IKK phosphorylation observed in ECs lacking AMPK-α2 but not -α1[96]. Alternatively, AMPK-mediated phosphorylation of the transcriptional co-activator p300 has been proposed to block acetylation and DNA binding by the p65 subunit of NFκB[146]. The idea that AMPK inhibits NFκB signalling is reinforced by functional studies demonstrating AMPK-dependent inhibition of NFκB-regulated expression of adhesion molecules and MCP-1[96, 144]. Fewer studies have investigated the effect of AMPK on proinflammatory JNK and IL-6 signaling, although AICAR and metformin reduce JNK activity in ECs[147], and increased JNK phosphorylation has been reported in ECs lacking AMPK-α2[95]. The mechanism of JNK inhibition is uncertain, although AMPK-dependent inhibition of the upstream kinase MKK4 has been reported in other cells[148]. Recently, AMPK-dependent inhibition of IL-6-stimulated JAK-STAT signaling has been demonstrated in ECs, potentially via direct inhibitory phosphorylation of JAK1 by AMPK[110]. AMPK activation therefore appears to rapidly suppress multiple proinflammatory signaling pathways in ECs, by diverse mechanisms.

AMPK activation in VSMCs inhibits TNFα-stimulated NFκB activity and angiotensin II-stimulated STAT1 activity, as well as reducing expression of inducible NOS and cyclooxygenase-2, and secretion of IL-6 and MCP-1[149, 150]. Thus, AMPK has anti-inflammatory effects in VSMCs as well as ECs.

AMPK and angiogenesis

Hypoxia, VEGF and adiponectin all stimulate AMPK-dependent EC migration, although there are conflicting reports as to whether this is mediated by NO[94, 98, 101, 105, 108]. Conversely, down-regulation of AMPK attenuates angiogenesis caused by hypoxia, adiponectin, VEGF or statins, in either tube formation or matrigel plug assays[94, 98, 101, 113]. Mechanistically, increased UCP2 or SOD2 have been reported to increase angiogenesis in AMPK-deficient ECs[151, 152], indicating that down-

regulation of ROS may be critical. AMPK activation also stimulates VEGF expression, indicating that AMPK positively influences angiogenesis both by increasing VEGF levels and by increasing VEGF signaling[153]. As angiogenesis would also consume significant amounts of ATP, AMPK activation might also serve a permissive role, ensuring adequate generation of ATP to permit EC migration and proliferation.

AMPK and VSMC contraction

AMPK has direct anti-contractile effects on VSMCs, because AICAR relaxes aortic rings in an NO- and endothelium-independent manner, an effect lost in AMPK-α1 knockouts[121]. Furthermore, AMPK activation has been reported to affect VSMC contractile signalling, including dephosphorylation of myosin light chain (MLC) and/or myosin phosphatase targeting subunit 1 (MYPT1)[125, 154]. Mechanistically, MLC/MYPT1 dephosphorylation may be a consequence of AMPK-mediated inhibition and phosphorylation of RhoA at Ser188, causing subsequent inhibition of ROCK[125]. A769662 has also been reported to reduce intracellular Ca^{2+} in VSMCs by increasing sarco/endoplasmic Ca^{2+} ATPase (SERCA) activity, associated with increased phosphorylation of phospholamban on Thr17, which disinhibits SERCA and thereby may underlie SERCA activation and vessel relaxation[155].

AMPK and proliferation, differentiation and migration of VSMCs

Unlike ECs, where AMPK up-regulates proliferation and migration thus supporting angiogenesis, in VSMCs AMPK inhibits proliferation in response to angiotensin II, PDGF and FCS, associated with stimulation of p53 Ser15 phosphorylation and reduced Rb phosphorylation[128, 156]. VSMCs from mice lacking AMPK-α2 exhibit increased proliferation, an effect mediated by increased degradation of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ triggered by the ubiquitin E3-ligase Skp2[157]. VSMCs retain significant plasticity *in vivo* and can exhibit a synthetic, proliferative phenotype rather than the quiescent, contractile phenotype observed during atherogenesis. AMPK not only suppresses VSMC proliferation, but also inhibits migration and maintains a pro-contractile phenotype[158, 159]. Inhibition of migration and proliferation are likely to be linked, because increased migration of VSMCs lacking AMPK-α2 is reported to be Skp2-dependent[159]. Furthermore, AICAR limits neointima formation after wire injury of rat femoral arteries, which is likely to reflect the anti-proliferative, anti-migratory actions of AMPK on VSMCs[127].

AMPK and VSMC calcification

As mentioned above, culture in high phosphate/β-glycerophosphate concentrations is used experimentally to stimulate calcium deposition in VSMCs. *In vivo*, such vascular calcification is frequently associated with ageing, atherosclerosis and diabetes mellitus. AICAR, adiponectin and metformin all inhibit VSMC calcification *in vitro*[130, 131, 160]. In recent studies of atherosclerosis-prone *ApoE*$^{-/-}$ mice, deletion of AMPK-α1 but not AMPK-α2 caused greater calcification of atherogenic plaques, and levels of the osteogenic transcription factor Runx2[160]. Furthermore, metformin reduced atherosclerotic calcification and Runx2 expression in the mice, an effect that was absent in ApoE$^{-/-}$ mice lacking AMPK-α1. The authors of that study further demonstrated that VSMC-specific AMPK-α1 deletion in ApoE$^{-/-}$ mice phenocopied the increased calcification and Runx2 expression, whereas macrophage-specific AMPK-α1 deletion had no effect. The mechanism underlying the AMPK-mediated inhibition of Runx2 levels was further proposed to be mediated by phosphorylation of PIAS1 (protein inhibitor of activated STAT-1) at Ser510, which acts as a SUMO E3-ligase to trigger Runx2 SUMOylation and degradation[160].

ROLE OF AMPK IN THE VASCULATURE IN VIVO

As many of the actions of AMPK in ECs and VSMCs should have beneficial effects on vascular function (Fig 5), considerable efforts have been made to examine whether AMPK influences vascular tone, remodelling and atherogenesis *in vivo*. Under physiological conditions, vascular AMPK has been reported to be activated *in vivo* by exercise[161] and estradiol[125], and is suppressed by high fat and fructose diets in rodents[162, 163]. Furthermore, exercise stimulates aortic AMPK phosphorylation in both ECs and VSMCs as assessed by immunohistochemistry[161].

AMPK and vascular tone

As described above, AMPK can stimulate NO synthesis by ECs and independently inhibit contractile protein function in VSMCs *in vitro*. In intact arterial vessels, AICAR stimulates vasodilation in a diverse range of vascular beds from several species[121, 164-167], an effect greatly attenuated in mice lacking AMPK-α1[121]. The mechanism of AICAR-stimulated vasodilation remains uncertain, and has been variously reported to be endothelium- and NO-dependent[164, 167], partially NO- and endothelium-dependent[165, 166] or NO- and endothelium-independent[121]. Using endothelium-specific knockouts it has been proposed that AMPK-α1 is important for endothelium-

518 dependent hyperpolarisation-mediated relaxation of resistance arteries[168]. Interestingly, resistance
519 arteries exhibited endothelium-independent dilation in response to A769662[155], suggesting a
520 VSMC-mediated effect. It is possible that ECs and VSMCs exhibit differential sensitivities to
521 AICAR and A769662, or that some of these may be AMPK-independent effects. Despite studies
522 indicating that AMPK-α2 has a less important role in regulation of vascular tone[121, 168], AMPK-α2
523 knockout mice are hypertensive and exhibit increased contractile responses to phenylephrine in
524 aortic rings[169]. Furthermore, impaired bradykinin-dependent vasodilation has been described in EC-
525 specific AMPK-α2 knockout mice, thought to be due to increased bradykinin degradation by
526 angiotensin-converting enzyme activity[170]. AICAR rapidly reduced blood pressure in spontaneously
527 hypertensive rats, but was without effect in normotensive controls[165], while prolonged
528 administration of AICAR reduced systolic blood pressure in obese Zucker rats[171]. These data
529 support a role for AMPK in the regulation of vascular tone in disease models, but cannot exclude
530 systemic actions on the heart, kidney or other tissues. Surprisingly, whether systemic administration
531 of more selective AMPK activators, such as A769662, 991 or C13, alters vascular tone has yet to be
532 reported. More recently it has become clear that perivascular adipose tissue (PVAT), which is
533 removed in most myography protocols, has a paracrine anti-contractile effect on the underlying
534 vessel. AMPK activity in resistance arteries has been shown to alter the influence of PVAT-derived
535 mediators[172] and the anti-contractile action of PVAT is absent in mice lacking AMPK-α1, perhaps
536 due to reduced adiponectin secretion[173]. AMPK in ECs, VSMCs and adventitial PVAT may
537 therefore all contribute to the maintenance of vascular tone, but the cell type that mediates actions
538 of AMPK on vascular tone may change, depending on the location of the vessel.

539 ***AMPK and atherosclerosis***

540 The potential anti-atherogenic actions of AMPK have been investigated in vascular injury models
541 and atherosclerosis-prone hypercholesterolemic mice. AICAR attenuates post-ischaemic leukocyte
542 rolling and adhesion to the endothelium *in vivo*, an effect lost in AMPK-α1 or AMPK-α2 knockout
543 mice[174], and systemic administration of AICAR or metformin reduces atherosclerotic lesion size,
544 macrophage accumulation and inflammation[175-177]. Similarly, berberine reduced the severity of
545 atherosclerotic lesions in atherosclerosis-prone mice, an effect attenuated in AMPK-α2
546 knockouts[178]. AMPKα2-deficient mice also exhibited increased atherosclerosis[95], although the

global deficiency makes it difficult to assess whether this was due to a direct effect on vascular tissues. With respect to plaque stability and progression, recent studies have yielded exciting results. Metformin reduced plaque calcification, and mice with a SMC-specific knockout of AMPK-α1 had exacerbated calcification of atherosclerotic plaques in brachiocephalic arteries, phenocopying the increased calcification observed in global AMPK-α1 but not AMPK-α2 knockouts[160]. As the clinical use of metformin is associated with reduced macrovascular morbidity and mortality independently of glycemia[179], these results may help to define the pathways involved. Using a ligation model for studying injury-induced neointima stability in brachiocephalic arteries, mice with deletion of AMPK-α1 but not AMPK-α2 exhibited more occlusive lesions, with lower collagen and higher macrophage content, indicative of plaque instability[180]. Similarly, in a high fat diet model, SMC-specific AMPK-α2 knockouts exhibited features of unstable plaques, including phenotype switching of VSMCs[158]. These data therefore indicate that AMPK activation may not only inhibit atherogenesis, but also inhibit the generation of vulnerable, calcified plaques. Repair of damaged endothelium is also considered important to prevent endothelial dysfunction after injury, and EC-specific expression of constitutively active AMPK has also been reported to promote re-endothelialization in a wire injury model, attributable in part to increased mobilization and incorporation of endothelial progenitor cells[181].

Immune cells are involved in all stages of atherosclerosis, and AMPK suppresses inflammatory signalling, monocyte-to-macrophage differentiation and foam cell formation[175, 177, 182]. Indeed, myeloid-specific AMPK-α1 knockout mice fed an atherogenic diet on a LDL receptor knockout background have recently been shown to exhibit exacerbated atherosclerosis, with increased plaque macrophage content and inflammatory gene expression[182]. All of these studies suggest that AMPK activation limits atherosclerosis, although all of the results are from rodent models, rather than humans. One intriguing question is how, despite only contributing a small fraction of total AMPK activity in vascular cells, specific down-regulation of AMPK-α2 has such marked effects on atheroma development in mice [95, 158, 174, 178], similar to the specific effects noted with respect to vascular tone[169, 170]. It remains to be determined whether there are isoform-specific substrates or specific subcellular localisations that contribute to the vascular function of AMPK-α2.

AMPK and pulmonary vascular remodelling

Several studies have identified a critical role for AMPK in the pulmonary vascular remodelling and perivascular inflammation that characterizes pulmonary arterial hypertension (PAH). Metformin reduces pulmonary artery VSMC proliferation in an AMPK-dependent manner[183], and inhibits the vascular remodelling of pulmonary arteries in rodent models of PAH[184, 185] . Mice with an EC-specific lack of AMPK also exhibit accelerated pulmonary remodeling[185]. Whether the beneficial action of metformin in PAH is mediated by AMPK remains to be determined. By contrast, AMPK activation by hypoxia may contribute to the development of PAH by promoting pulmonary VSMC survival[186], while AMPK-mediated inhibition of the voltage-gated K^+ channel Kv1.5 may underlie the acute detrimental effects of hypoxia on PAH[187].

Conclusions and Perspectives

AMPK exists as heterotrimeric complexes consisting of catalytic α subunits and regulatory β and γ subunits. AMPK complexes sense the energy status of cells by sensing increases in the cellular AMP:ATP and/or ADP:ATP ratios. AMP, ADP and ATP bind at up to three sites on the γ subunits; binding of AMP and/or ADP causes activation by promoting net phosphorylation at Thr172 within the activation loop on the α subunit, while binding of AMP only causes further allosteric activation. Once activated by energy stress, AMPK switches on catabolic pathways that generate ATP, while switching off cell growth and proliferation and other processes that consume ATP.

AMPK appears to exert a protective effect in rodent heart during ischemic episodes. Inherited and/or *de novo* mutations in the *PRKAG2* gene (encoding AMPK-γ2) in humans cause heart disease of varying severity characterized by ventricular pre-excitation, excessive cardiac glycogen content, and hypertrophy. The mutations cause an increase in basal AMPK activity, leading to increased glucose uptake that accounts for the first two abnormalities. They also cause a failure of γ2-containing complexes to be further activated by AMP, which might explain the hypertrophy through unrestrained activity of the mTORC1 pathway.

In blood vessels, AMPK inactivation is associated with anti-contractile, anti-inflammatory and anti-atherogenic actions on both the vascular endothelium and smooth muscle. Exciting recent studies link AMPK activation to increased stability and reduced calcification of atherosclerotic plaques as well as highlighting a potential role for AMPK in pulmonary arterial hypertension.

Given the critical role of AMPK in the regulation of nutrient metabolism, therapies that activate AMPK may not only normalise metabolic dysfunction but also reduce the burden of cardiovascular complications in obesity and type 2 diabetes. Recent studies using vascular tissue-specific deletion of AMPK-α isoforms are beginning to elucidate specific roles for AMPK-α isoforms, yet despite the wealth of research demonstrating the functional cardiovascular consequences of AMPK activation, the AMPK substrates involved and underlying mechanisms in several cases remain poorly defined. Emerging technologies such as phosphoproteomics may prove beneficial in understanding such mechanisms after AMPK up- or down-regulation in cardiovascular tissues in different disease settings and disease models.

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FIGURE LEGENDS

Figure 1: Domain layouts of AMPK subunits and their isoforms. The linear layout of domains is shown, approximately to scale and with similar color coding as in Fig. 2. Note that both β subunits are N-myristoylated, and that the γ2 and γ3 subunit isoforms have unrelated N-terminal extensions of unknown function, although both are also reported to exist as shorter, N-terminally truncated versions due to alternate start sites and/or splicing[188].

Figure 2: Structure of human AMPK (α1β2γ1 complex)[7]. The model was created in spacefilling mode using PyMol v1.7.4.2 with the co-ordinates in PDB file 4RER, and with color coding similar to Fig. 1. The heterotrimer was crystallized in the presence of β-cyclodextrin, which occupies the glycogen-binding site, staurosporine, which occupies the active site, and AMP, which occupies sites 1, 3 and 4 on the γ subunit (sites 1 and 4 are round the back in this view). Although Thr172 was phosphorylated, it is not visible in this view but lies in the cleft between the α subunit C lobe and the β-CTD, just over the right-hand "shoulder" of the C-lobe.

Figure 3: Two views of the structure of the four CBS repeats of the γ1 subunit. The model used the co-ordinates in PDB file 2V8Q[18], and was rendered in PyMol v1.7.4.2 with the γ1 subunit in cartoon view. The two views are rotated 180^{o} around the x axis (dashed line) with respect to each other, with the orientation of the top view being similar to that in Fig. 2. Note the pseudosymmetrical layout of the four CBS repeats, which are colored differently (and differently to Figs. 1 and 2). Residues equivalent to those mutated in γ2 are highlighted using the "dots" version of space-filling representation, and are numbered using the human γ2 numbering. The three molecules of bound AMP are labelled and are shown in standard space-filling view, with C atoms green, O red and N blue.

Figure 4: Regulation of endothelial NO and superoxide synthesis by AMPK. AMPK activation stimulates NO synthesis via multiple mechanisms: (i) phosphorylation of eNOS at Ser633 and Ser1177; (ii) increasing Hsp90 association with eNOS; (iii) increasing BH_4 concentrations via GTP cyclohydrolase I (GTPCH1); (iv) reducing superoxide synthesis via inhibition of Nox and increasing antioxidant protein

(superoxide dismutase (SOD), thioredoxin and catalase) levels. NO itself is also reported to activate AMPK.

Figure 5: Actions of AMPK in vascular cells. Physiological activators of AMPK in ECs include hypoxia/ischemia, shear stress, adiponectin, thrombin, bradykinin and VEGF (synthesis of which is stimulated by AMPK in other tissues). AMPK is reported to stimulate VSMC relaxation through: (i) increased NO synthesis and possibly endothelium-dependent hypopolarising factor (EDHF); (ii) inhibition of MYPT1/MLC phosphorylation and Ca^{2+} levels in VSMCs, reported to be mediated by reduced RhoA activity and increased sarco/endoplasmic Ca^{2+} ATPase (SERCA) activity respectively. AMPK activation in ECs stimulates proliferation and migration, whereas in VSMCs, proliferation and migration are inhibited, associated with p53 phosphorylation, Rb dephosphorylation and p27(Kip1) stabilisation. AMPK also inhibits VSMC calcification by reducing Runx2 and pro-inflammatory signalling pathways leading to leukocyte adhesion and cytokine/chemokine synthesis.

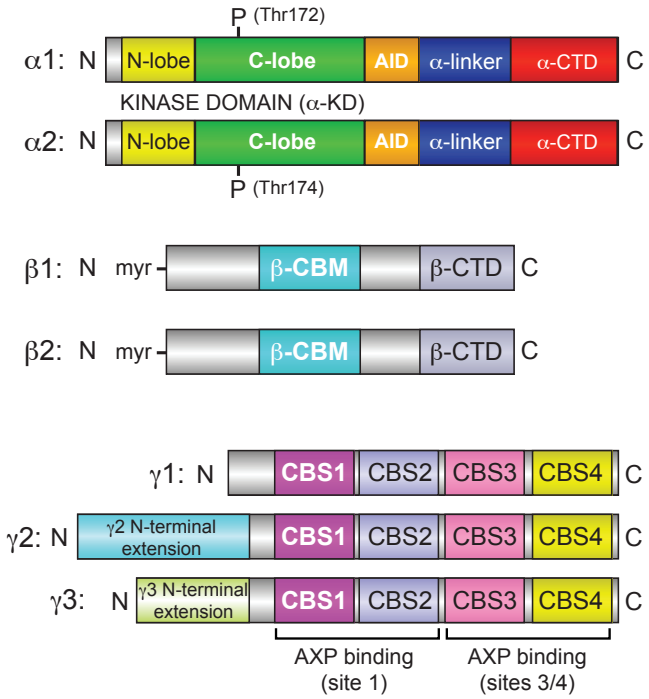
Table 1: List of pharmacological agents commonly used to activate AMPK in intact cells or in vivo, and their mechanisms of action.

Class	Agent	Mechanism	Binding site used	Isoform-selective?	Ref.
1	metformin	mitochondrial inhibitor, AMP ↑	γ subunit (binds AMP)	No	[33]
1	phenformin	mitochondrial inhibitor, AMP ↑	γ subunit (binds AMP)	No	[33]
1	berberine	mitochondrial inhibitor, AMP ↑	γ subunit (binds AMP)	No	[33]
2	2-deoxyglucose	glycolytic inhibitor, AMP ↑	γ subunit (binds AMP)	No	[33]
3	AICAR	pro-drug, converted to ZMP	γ subunit (binds ZMP)	No	[33, 34]
4	C13	pro-drug, converted to C2	γ subunit (binds C2)	α1-selective	[38, 40]
5	A769662	direct activator	ADaM site	β1-selective	[189]
5	991	direct activator	ADaM site	β1>β2	[8]
5	MT 63-78	direct activator	ADaM site	β1-selective	[190]
5	PF-06409577	direct activator	ADaM site	β1-selective	[191]
5	PF-249	direct activator	ADaM site	β1-selective	[191]
5	salicylate	direct activator	ADaM site	β1-selective	[9, 44]

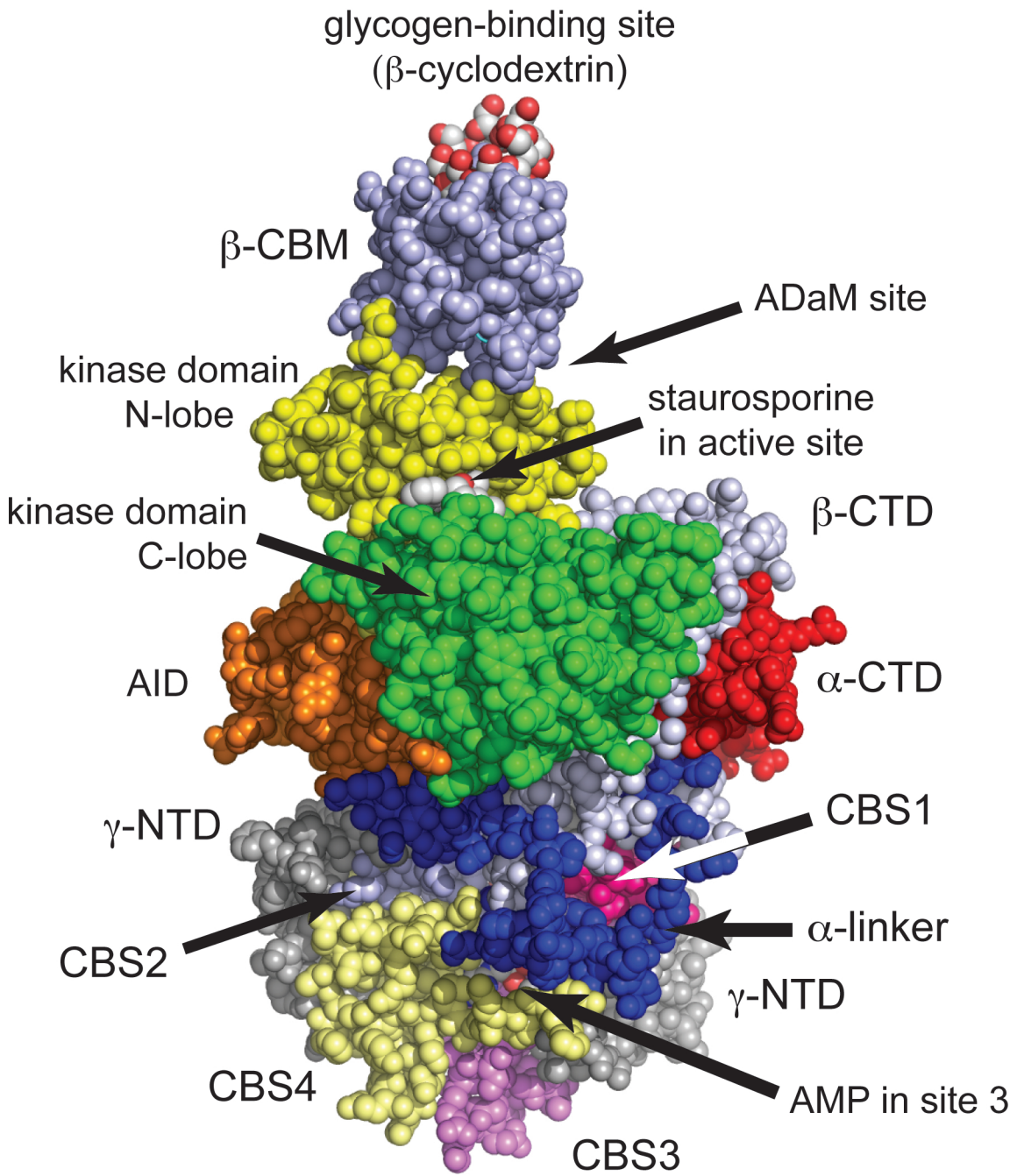
Table 2: Amino acid replacements, generated by mutations in *PRKAG2,* that are associated with heart disorders. Equivalent residues in the sequences of human γ1 and γ3 are shown in the second and third column and the CBS repeat affected in the fourth. V336 and R350 are located in the linker between CBS1 and CBS2.

γ2 mutation	γ1 equivalent	γ3 equivalent	CBS repeat affected	Reference
R302Q	R70	R225	CBS1	192-194
S333P	S101	S333	CBS1	79
V336A	V104	V259	linker	79
L insert (after R350)	after R118	after R273	linker	195
H383R	H151	H306	CBS2	195
R384T	R152	R307	CBS2	77
T400N	T168	T323	CBS2	193
Y487H	Y255	Y410	CBS3	196
N488I	N256	N411	CBS3	193
E506K	E274	E429	CBS4	79, 197
H530R	H298	H453	CBS4	198
R531G	R299	R454	CBS4	80
R531Q	R299	R454	CBS4	78
S548P	S316	S471	CBS4	199

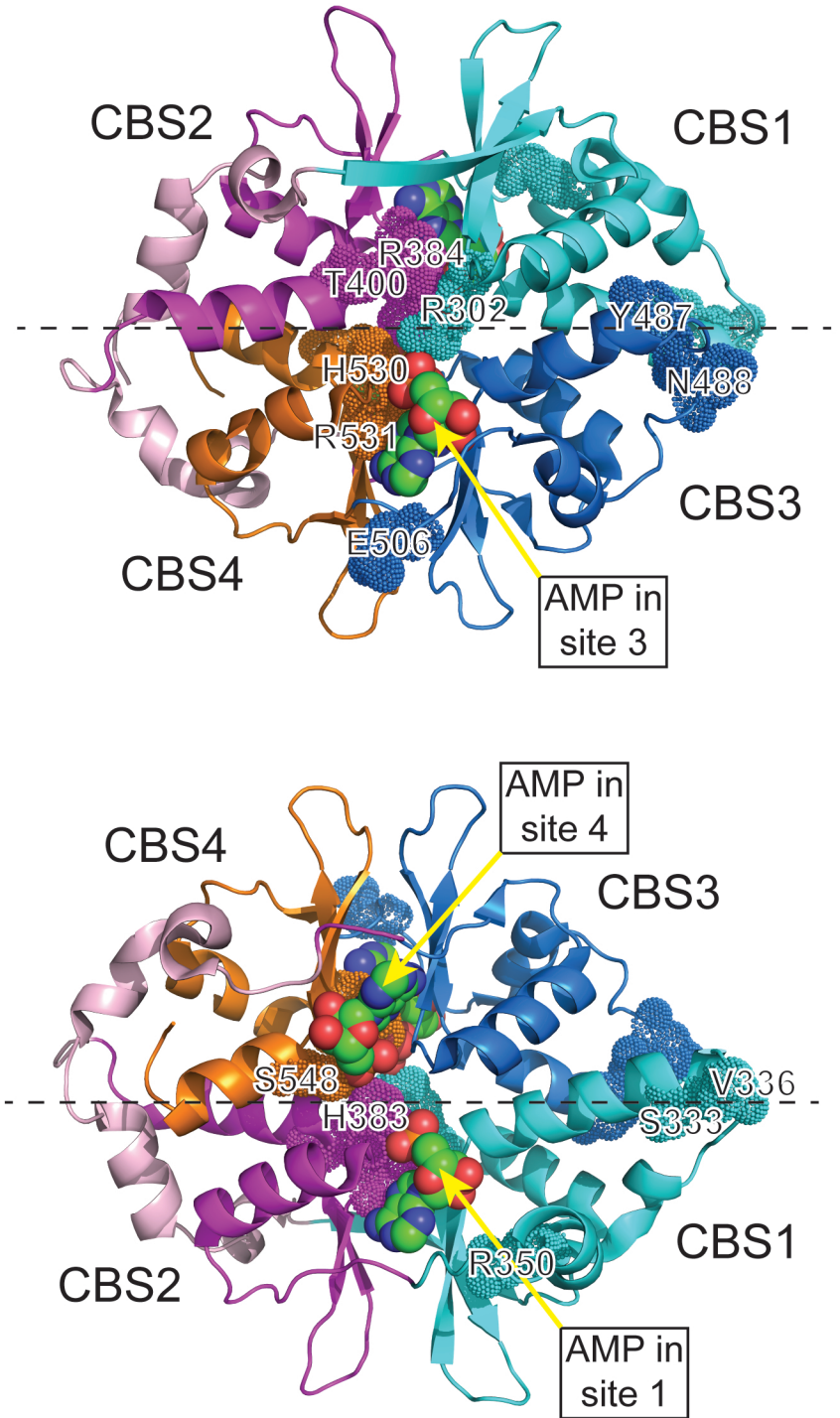
Salt & Hardie Fig. 1



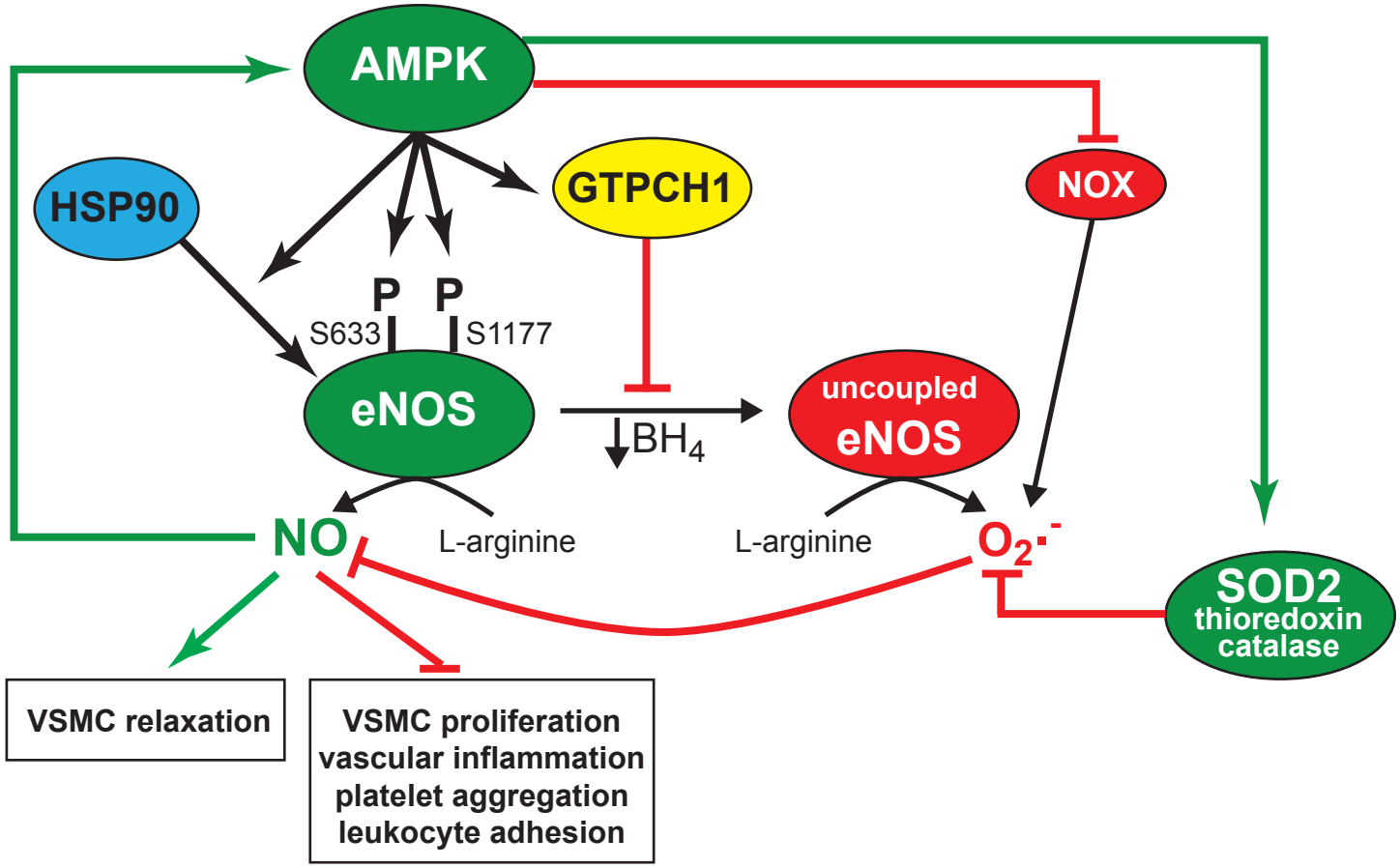
Salt & Hardie Fig. 2



Salt & Hardie Fig. 3



Salt & Hardie Fig. 4



Salt & Hardie Fig. 5